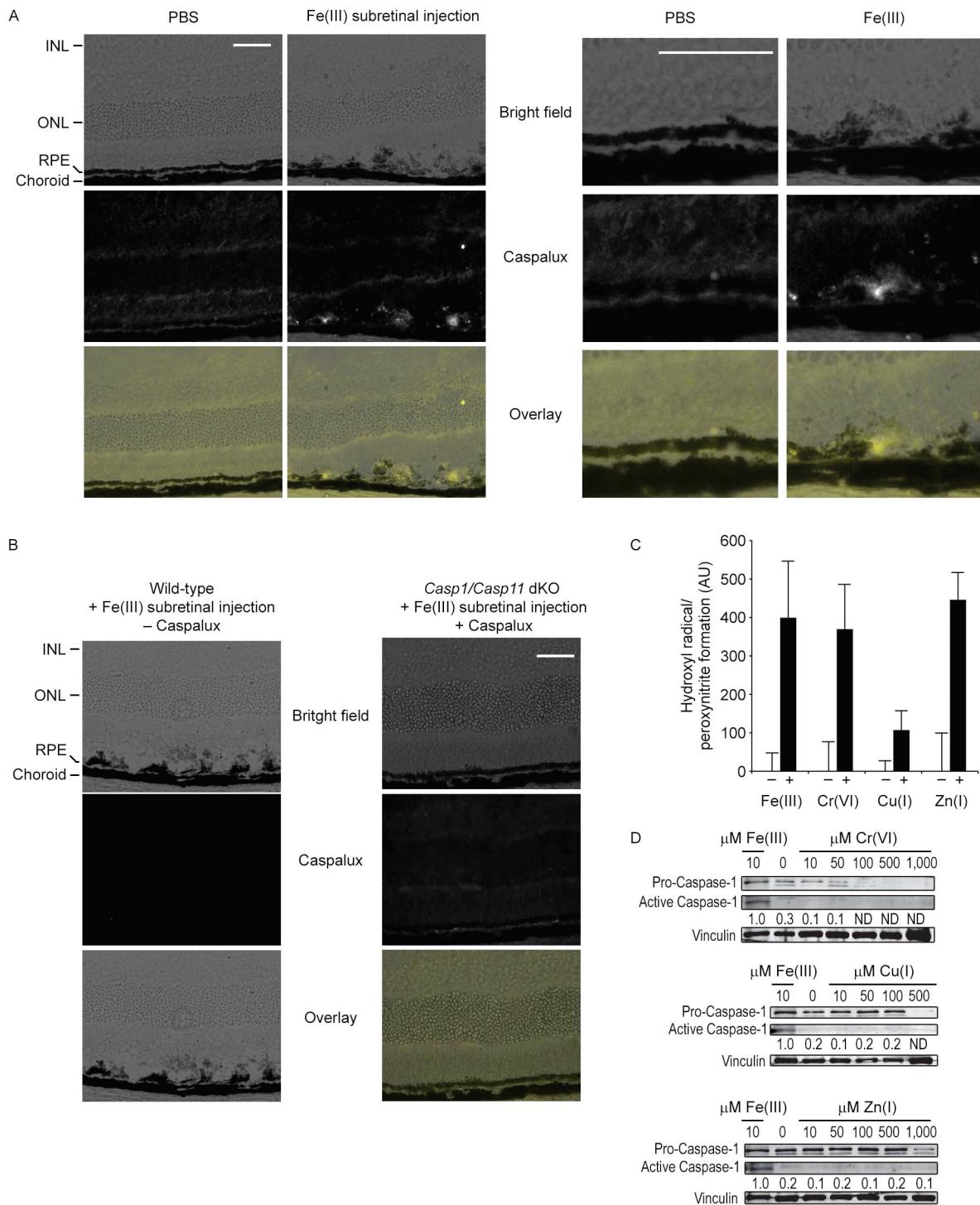


## **Supplemental Figures**

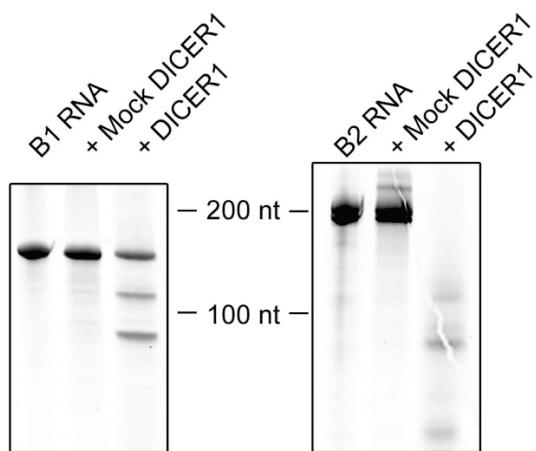
**Figure S1, related to Figure 1**



(A) Bright-field and Caspase-1 reporter peptide visualization of wild-type mouse retinal cross sections after saline or 100 nM Fe(III) injection. Scale bars denote 50  $\mu$ m. (B, left) Bright-field

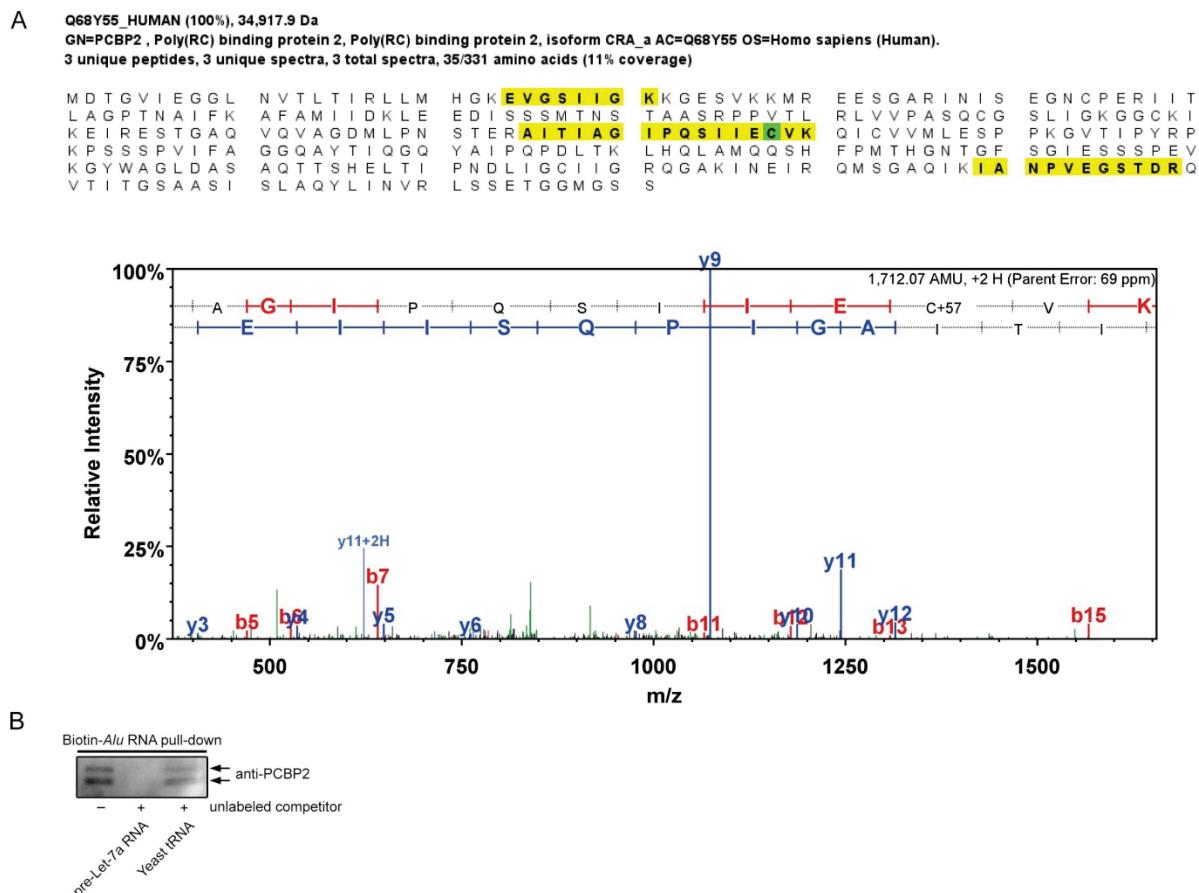
and fluorescent imaging of wild-type retinal cross sections without administration of Caspase-1-sensitive fluorescent peptide. **(B, right)** Retinal cross sections from iron-treated Caspase-1/11 knockout mice exposed to Caspase-1-sensitive fluorescent peptide. Scale bar denotes 50  $\mu$ m. **(C)** Detection of hydroxyl radical formation after treatment with Fenton metals: 1 mM Fe(III), 1 mM Cr(VI), 0.5 mM Cu(I) or 1 mM Zn(I). N=4, error bars denote SEM. **(D)** Western blotting of human ARPE-19 cells treated with indicated doses of Fe(III), Cr(VI), Cu(I) or Zn(I). N=3.

**Figure S2, related to Figure 2**



Polyacrylamide gel separated B1 and B2 RNAs after incubation with recombinant DICER1 or enzyme-free (mock) reaction.

**Figure S3, related to Figure 4**



(A) Alignment, coverage and mass spectrum of PCBP2 obtained from mass spectrometry analysis of *Alu* RNA-bound proteins enriched from human RPE cell lysates.

(B) Western blotting of streptavidin-mediated pull-down from biotin-*Alu* RNA transfected in the presence of either unlabeled pre-Let-7a or unlabeled yeast tRNA into human ARPE-19 cells.

## **Supplemental Experimental Procedures**

### **Animal Husbandry**

All animal experiments were approved by institutional review committees and in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. Wild-type C57BL/6J mice were purchased from The Jackson Laboratory. Casp1/11<sup>-/-</sup> and Nlrp3<sup>-/-</sup> mice have been previously described (Kanneganti et al., 2006). For all procedures, anesthesia was achieved by intraperitoneal injection of 100 mg/kg ketamine hydrochloride (Ft. Dodge Animal Health) and 10 mg/kg xylazine (Phoenix Scientific), and pupils were dilated with topical 1% tropicamide (Alcon Laboratories). Fundus imaging was performed on a TRC-50 IX camera (Topcon) linked to a digital imaging system (Sony).

### **Intraocular injection**

Subretinal injections and intravitreous injections (1  $\mu$ L each) were performed with a 35-gauge Exmire microsyringe (Ito Corporation). Fe(III) ammonium citrate, Cr(VI) oxide, Cu(I) sulfate and Zn(I) chloride were dissolved in sterile phosphate buffered saline at indicated concentrations, or an equal volume of sterile phosphate buffered saline, and delivered via subretinal injection 6-7 days prior to analysis. Cell-permeating cholesterol conjugated B2 antisense oligonucleotide (AS) (5'-TCAGATCTCGTTACGGATGGTTGTGA-3') or cholesterol conjugated-control AS (5'-TTGGTACGCATACGTGTTGACTGTGA-3') (both from Integrated DNA Technologies, IDT) were resuspended in sterile phosphate buffered saline and injected (1  $\mu$ g in 1  $\mu$ L) into the vitreous cavity of wild-type mice 24 h prior to iron injection.

### ***In vitro* transcription and labeling**

Transcription of *Alu*, B1 and B2 RNAs was performed using linearized templates described previously (Tarallo et al., 2012) using AmpliScribe T7-Flash Transcription Kit (Epicentre) according to the manufacturer's instructions. DNase-treated RNA was purified using MEGAclear™ (Ambion), and integrity was monitored by gel electrophoresis. For biotin labeling, transcription reactions were performed using AmpliScribe- T7-Flash-Biotin-RNA Transcription Kit (Epicentre). Yeast tRNA was labeled with biotin using the 3' End Biotinylation Kit (Life Technologies) according to the manufacturer's instructions.

### **Cell culture**

Human ARPE-19 cells were purchased from ATCC, and maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum and standard antibiotic concentrations. Mouse embryonic fibroblasts were purchased from ATCC and maintained in DMEM supplemented with 10% FBS and antibiotics. Overload of metal ions was achieved by supplementing the culture medium with indicated concentrations of Fe(III) ammonium citrate, Cr(VI) sulfate, Cu(I) chloride and Zn(I) chloride for 72-96 hr.

### **Transfection**

All transfections unless otherwise indicated were performed using Lipofectamine2000 (Life Technologies) according to the manufacturer's instructions. Antisense oligonucleotides for DICER1 and *Alu* RNA (and controls) were synthesized by IDT. (5'- DICER1 antisense

oligonucleotide (AS) (5'-GCUGACCTTTGCTUCUCA-3'), control (for DICER1) AS (5'-TTGGTACGCATACGTGTTGACTGTGA-3'), Alu AS (5'-CCCGGGTTCACGCCATTCTCCTGCCTCAGCCTCACGAGTAGCTGGGACTACAGGCGCCCGACACCACCTCCGGCTAATTTTGTTATT-3'), control (for Alu) AS (5'-GCATGGCCAGTCCATTGATCTTGCACGCTGCCTAGTACGCTCCTAACCTATCCTCTAGCCCCGTTACTTGGTGCCACCGGCG-3').

### **Northern blotting**

Total RNA was extracted by TRIzol (Life Technologies) and separated on TBE-urea polyacrylamide gels, transferred to Hybond membranes (Amersham) and hybridized with biotinylated DNA probes for indicated targets. Probes for B1, B2 U6 and 5S RNAs were synthesized by IDT. B1 (5'-TTCTCTGTGTAGCCCTGGCTGCCTGGAACTCACT-3'), B2 (5'-TGAGCCACCATGTGGTTGCTGGAAATTGAACTCAG-3'), U6 (5'-CACGAATTGCGTGTACCTT-3'), 5S (5'- AGCCTACAGCACCCGGTATT-3'). For *Alu* RNA northern blotting, biotinylated probe was synthesized by PCR of an *Alu*Ya5 sequence described previously (Shaikh et al., 1997). Probes were hybridized at 42 °C overnight and visualized using the Chemiluminescent Nucleic Acid Detection Module (Thermo).

### **Western blotting**

Total protein lysates were subjected to SDS-PAGE and transferred to PVDF membranes. Antibody detection was performed by either HRP-based chemiluminescent system or infrared fluorescent scanning (Licor).

### **Cell-based *Alu* RNA degradation assay**

Biotin-labeled *Alu* RNA was transfected into cells for 2 h using Lipofectamine2000 (Invitrogen). After 2 h, cells were thoroughly washed, and collected at indicated times in TRIZOL. RNA was purified, run on a 10%TBE-urea polyacrylamide gel, transferred to a membrane and visualized using Chemiluminescent Nucleic Acid Detection Module (Thermo). Afterwards, the membranes were probed for control genes (U6 and/or 5S RNAs). Full-length (e.g. 300 nt) *Alu* RNA and control genes were quantified using densitometry analysis in ImageJ.

### **Run-on assay**

Native *Alu* RNA transcription and degradation rates were evaluated using Click-It Nascent RNA Capture Kit (Life Technologies) according to the manufacturer's instructions, with minor modifications. Briefly, cells pre-loaded with iron (or control cells) were incubated with Ethylene uridine for 4 hours, then either lysed in TRIzol, or washed thoroughly and collected in TRIzol after 20 more hours. Total RNA was then extracted and separated on a TBE-urea polyacrylamide gel. To exclude *Alu* RNA signals from longer, embedded transcripts, RNA was extracted from 75-800 nt using a scalpel blade and RNA ladder as a guide. Ethylene uridine containing RNA was then extracted from the gel using the 'crush and soak' method, precipitated and the Click-It Nascent RNA Capture Kit was followed, allowing for biotinylation of RNAs, purification by streptavidin beads. DNase treatment and reverse transcription was performed using Quantitect Reverse Transcription Kit (Qiagen), and *Alu* and 5S RNAs were quantified by real-time RT-PCR using an Applied Biosystems 7900 HT Fast Real-Time PCR system by the  $2^{-\Delta\Delta C_t}$  method.

### ***In vitro* DICER1 reactions**

Expression and purification of human PCBP2 was performed as described previously (Li et al., 2012). Synthetic B1 and B2 RNAs were subjected to recombinant human DICER1 enzymatic cleavage (Genlantis) at 37 °C for 24 hours according to the manufacturer's instructions. Mock digestions were identical except that the DICER1 enzyme was omitted. Following incubation, RNAs were separated on by denaturing PAGE, and visualized by GelStar Nucleic Acid Gel Stain (Life Technologies). For quantitative determination of DICER1 enzymatic activity in the presence of iron, DICER1, and/or PCPB2, synthetic *Alu* RNA was incubated for 2 hours at 37 °C. Cleavage product (RNA fragments < 200 nt) was purified by 96-well RNA extraction column (Omega Bio-Tek) and product concentration measured by RiboGreen fluorescence (Life Technologies).

### **Pull-down assays**

Cells transfected with biotin-labeled *Alu* RNA or yeast tRNA were lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0.), then incubated with streptavidin-Dynabeads (Life Technologies) and a magnet was used to separate proteins that were bound and unbound to *Alu* RNA. Proteins were analyzed by western blotting (as described above). For antibody-based pull-downs (RNA immunoprecipitations), cells were lysed and processed using the MagnaRIP kit (Millipore). Mouse anti-PCBP2 (Abnova) or mouse IgG2ka control (Abcam) were utilized for pull-downs. RNAs were removed from the beads by TRIzol, and analyzed by northern blotting.

### **Mass-Spectrometry Assisted Identification of *Alu* RNA Protein Binding Partners**

To prepare Alu RNA protein binding partners for mass spectrometry analyses, we developed a whole body biotin labeled Alu RNA (bio-Alu) using T7 polymerase reverse transcription (Epicentre) from an Alu encoding plasmid. Protein lysates (500 µg in 10 mM Tris-HCl pH 7.4 with 10 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.1 mM DTT, 0.5% NP40, 200 µg/mL tRNA, 20 units of DNase I, 20 units RNase Inhibitor) were prepared from primary human RPE isolates (Lonza Biosciences) and incubated with the bio-Alu probe (3µg) for 1 hr at 25°C. Streptavidin coupled magnetic beads (Dynabeads, Life Technologies) were then added followed by incubation for 1 hr at 4°C and bead separation with 4 washes. Bound protein was eluted (in 10mM Tris-Cl pH 6.0 with 1mM EDTA, 2.0M NaCl, 0.5M MgCl<sub>2</sub>) and checked for quality and yield via PAGE and Coomassie Brilliant Blue. The composition of the Alu RNA binding proteins was analyzed by 2D liquid chromatography tandem mass spectrometry as previously described (MacCoss et al., 2002). Briefly, trypsin digested peptides were assessed with MudPIT (Multidimensional Protein Identification Technology) with a linear ion trap mass spectrometer (LTQ). Peptide spectral data was searched against a protein database using Sequest (Yates et al., 1995) and the resulting identifications collated and filtered using IDPicker (Ma et al., 2009) and Scaffold 3 (Proteome Software). Relative protein abundances were evaluated via spectral counting techniques using the Quasitel program for P-value calculations (Li et al., 2010).

### ***In situ* detection of Caspase-1 activity**

Eyes from treated animals were enucleated and immediately placed in OCT mounting media and snap frozen in isopentane cooled by liquid nitrogen. Unfixed 5 µm thick frozen sections of mouse eyes were incubated with CaspaLux1-E1D2 (Oncoimmunin) for 40 minutes at 37 °C in a humidified chamber. Afterwards, slides were washed 5 times in PBS. Coverslips were placed on

the tissue sections and fluorescent and bright-field images were acquired on a Nikon Eclipse Ti inverted fluorescent microscope.

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